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Molecular Identification of Black-Grain Mycetoma Agents

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Black-grain mycetomas are subcutaneous devastating chronic infections due to several dematiaceous fungi. They are diagnosed mostly in tropical countries. Identification of these fungi with standard mycological procedures is difficult because of their poor or delayed sporulation. The aim of this study was thus to assess the accuracy of molecular identification of these fungi. A total of 54 strains, mostly of clinical origin, were used, including 15 *Madurella mycetomatis*, 6 *Madurella grisea*, 12 *Leptosphaeria senegalensis*, 4 *Leptosphaeria tompkinsii*, 6 *Pyrenochaeta* spp., 4 *Curvularia lunata*, and 7 *Exophiala jeanselmei* strains. The internal transcribed spacer 1 (ITS1)-5.8S-ITS2 DNA region was amplified by using universal fungal primers and sequenced. Both intra- and interspecies sequence similarities were assessed. *Madurella mycetomatis* appeared to be a homogeneous species. More intraspecies variations were found for *C. lunata* and *E. jeanselmei*, leading, in some instances, to changes in the initial identification. *L. senegalensis* and *L. tompkinsii* showed intraspecies similarities of >99%, but similarity between the two species was <88%. Intergenera and interspecies variations were important, with sequence homologies of <81% between genera. In contrast, *Pyrenochaeta romeroi* and *M. grisea* appeared to be heterogeneous, with intraspecies similarities of 40 to 100% and 53 to 100%, respectively, which suggest either erroneous identification or the need for taxonomic revision. Epidemiological and therapeutic studies could benefit from a precise identification of the fungi responsible for black-grain mycetoma based not only on phenotypical characteristics but also on ITS sequencing.

Tropical mycetoma is a highly debilitating disease characterized by a subcutaneous mass with multiple sinuses draining pus and "grains" defining the typical lesion. This chronic infection is thought to start following a subcutaneous inoculation of the microorganisms and remains indolent until extension to deeper tissues and bone occurs (10). In countries where mycetoma is endemic, patients seek medical advice often years after the first symptoms, at a time when efficient surgery is often unrealistic and/or mutilating. For most of these patients, there is an unmet need for adequate medical treatment. The first step is thus to distinguish between infection caused by true fungi (eumycetoma) and that caused by actinomycetes (actinomycetoma). Along with clinical and epidemiological data (4), the differential diagnosis relies on the morphological characteristics of the grains. These grains are aggregates of fungal hyphae or gram-positive bacterial filaments embedded in tough, cement-like material.

Black-grain mycetomas are caused by fungi only. However, the etiologic agents are very diverse (15), and their frequency depends mainly on the geographical area and thus the climatic environment. Indeed, eumycetoma, initially described in India (Madura foot), has since been recognized to have a worldwide distribution, between latitudes 15°S and 30°N (4, 10). Africa is the continent most concerned, from Sudan to Senegal, delineating the "mycetoma belt."

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Identification of the various dematiaceous fungi responsible for black-grain mycetoma remains difficult with standard mycological procedures and can be delayed for up to 12 weeks (7). Cultures are often negative or contaminated with bacteria. When the culture is positive, the morphological features are often poorly differentiated and therefore not reliable for species identification. Therefore, the need for reliable tools to improve the diagnosis, epidemiological investigations, and treatment evaluation of eumycetoma has led to the development of molecular tests. A species-specific PCR test has been shown to be useful for the identification of *Madurella mycetomatis*, the main species responsible for eumycetoma (2, 3). This has lead to a modification of the taxonomic position of *M. mycetomatis* and differentiates it from *M. grisea* (5). We therefore thought it useful to perform an exhaustive analysis of small-subunit rRNA genes and internal transcribed spacer (ITS) sequences of the various species responsible for black grain mycetoma. We used the different strains available at the Pasteur Institute, Paris, France, to look for polymorphisms within individual species and to assess the phylogenetic relationships between the different fungal species responsible for black-grain mycetoma.

MATERIALS AND METHODS

Strains. A total of 54 strains, known agents of black-grain mycetoma, from the Pasteur Institute (IP) collection of fungi as well as from the collection of the National Reference Center for Mycoses and Antifungals (CNRMA) were tested. Seventy-eight percent of these strains were of clinical origin. The majority of these strains were isolated from mycetoma lesions, and the geographical origins of the patients were diverse (Table 1). These strains included 15 *Madurella mycetomatis*, 6 *M. grisea*, 12 *Leptosphaeria senegalensis*, 4

TABLE 1. Characteristics of strains used in this study

Species[a]	Strain[b]	Source	Origin (reference)	GenBank accession no.	Final identification
Madurella mycetomatis[T]	CBS 109801	Mycetoma, foot	Sudan (5)	DQ836767	*Madurella mycetomatis*
Madurella mycetomatis	IP 582.60	Mycetoma	Chad (18)		*Madurella* sp.
Madurella mycetomatis	IP 584.78	Mycetoma	Morocco		*Madurella* sp.
Madurella mycetomatis	IP 591.78	Mycetoma	Morocco	DQ836768	*Madurella mycetomatis*
Madurella mycetomatis	IP 592.74	Mycetoma	Morocco		*Madurella* sp.
Madurella mycetomatis	IP 595.60	Mycetoma	Chad (18)		Unidentified fungal sp.
Madurella mycetomatis	IP 599.60	Mycetoma	Djibouti		Unidentified fungal sp.
Madurella mycetomatis	IP 1137.76	Mycetoma	NA		*Madurella* sp.
Madurella mycetomatis	IP 1175.79	Mycetoma	Cameroon	DQ836769	*Madurella mycetomatis*
Madurella mycetomatis	IP 1435.83	NA[c]	NA	DQ836770	*Madurella mycetomatis*
Madurella mycetomatis	IP 2299.95	Mycetoma	Niger	DQ836771	*Madurella mycetomatis*
Madurella mycetomatis	IP 2303.95	Mycetoma	Senegal		Unidentified fungal sp.
Madurella mycetomatis	CNRMA 03.683	Mycetoma, foot	Niger	DQ836772	*Madurella mycetomatis*
Madurella mycetomatis	CNRMA 03.978	NA	NA	DQ836773	*Madurella mycetomatis*
Madurella mycetomatis	CNRMA 03.1151	Mycetoma, foot	Niger	DQ836774	*Madurella mycetomatis*
Leptosphaeria senegalensis[T]	IP 614.60 (CBS 196.79, ATCC 18282)	Mycetoma	Senegal (17)	DQ836775	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 610.60	Mycetoma	Senegal	DQ836776	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 611.60 (ATCC 18262)	Mycetoma	Senegal	DQ836777	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 612.60	Mycetoma	Senegal	DQ836778	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 615.60	Mycetoma	Senegal	DQ836779	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 617.60 (CBS 197.79, ATCC 18264)	Mycetoma	Senegal	DQ836780	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 618.60	Mycetoma	Senegal	DQ836781	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 619.60 (CBS 198.79, ATCC 18265)	Mycetoma	Senegal	DQ836782	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 1092.74 (CBS 199.79)	Mycetoma	Senegal	DQ836783	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 1155.77	Mycetoma	Senegal	DQ836784	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 1259.81	Mycetoma	Senegal	DQ836785	*Leptosphaeria senegalensis*
Leptosphaeria senegalensis	IP 1766.88	Mycetoma	France	DQ836786	*Leptosphaeria senegalensis*
Leptosphaeria tompkinsii[T]	IP 559.60 (ATCC 16412)	Mycetoma	Mauritania (9)	DQ836787	*Leptosphaeria tompkinsii*
Leptosphaeria tompkinsii	IP 1151.76 (CBS 201.79)	Mycetoma	Senegal	DQ836788	*Leptosphaeria tompkinsii*
Leptosphaeria tompkinsii	IP 1156.77	Mycetoma	NA	DQ836789	*Leptosphaeria tompkinsii*
Leptosphaeria tompkinsii	CNRMA 04.1034	Mycetoma, foot	Niger	DQ836790	*Leptosphaeria tompkinsii*
Exophiala jeanselmei[T]	IP 71.52 (CBS 507.90, ATCC 34123)	Mycetoma	West Indies	DQ836791	*Exophiala jeanselmei*
Exophiala jeanselmei	IP 69.52	NA	NA	DQ836792	*Exophiala oligosperma*
Exophiala jeanselmei	IP 70.52	NA	NA	DQ836793	*Exophiala jeanselmei*
Exophiala jeanselmei	IP 1535.84	Bronchial fluid	France	DQ836794	*Exophiala oligosperma*
Exophiala jeanselmei	IP 1792.88 (CBS 528.76, ATCC 10224)	Mycetoma, hand	NA	DQ836795	*Exophiala jeanselmei*
Exophiala jeanselmei	IP 1908.90	Mycetoma	Cote d'Ivoire	DQ836796	*Exophiala oligosperma*
Exophiala jeanselmei	IP 2118.92	NA	France	DQ836797	*Exophiala oligosperma*
Curvularia lunata	IP 613.60	Mycetoma	Senegal	DQ836798	*Curvularia lunata*
Curvularia lunata	IP 1417.82	Saprophyte	NA	DQ836799	*Curvularia lunata*
Curvularia lunata	IP 1477.83	NA	France		*Curvularia* sp.
Curvularia lunata	IP 2328.95	Onyxis	West Indies	DQ836800	*Curvularia lunata*
Pyrenochaeta romeroi[T]	IP 862.63 (CBS 252.60, ATCC 13735)	Mycetoma, foot	Venezuela	DQ836801	*Pyrenochaeta romeroi*
Pyrenochaeta romeroi	IP 571.61	Mycetoma	Senegal	DQ836802	*Pyrenochaeta romeroi*
Pyrenochaeta romeroi	IP 888.65	Mycetoma	NA	DQ836803	*Pyrenochaeta romeroi*
Pyrenochaeta romeroi	IP 234.61	Mycetoma	Senegal		Unidentified fungal sp. 1
Pyrenochaeta sp.	IP 696	NA	NA		Unidentified fungal sp. 1
Pyrenochaeta romeroi	IP 1034.71	Mycetoma	Costa Rica		Unidentified fungal sp. 2
Madurella grisea	IP 1134.76	Mycetoma	South Africa		Unidentified fungal sp. 1
Madurella grisea	IP 67.52	NA	NA		Unidentified fungal sp. 2
Madurella grisea	IP 72.65	NA	United Kingdom		Unidentified fungal sp. 2
Madurella grisea	IP 66.52	NA	NA		Unidentified fungal sp. 3
Madurella grisea	IP 970.68	Mycetoma, foot	France		Unidentified fungal sp. 4
Madurella grisea	CNRMA 02.717	NA	West Indies		*Phomopsis* sp.

[a] T, type strain.
[b] IP, Pasteur Institute Collection of Fungi, Pasteur Institute, Paris, France; ATCC, American Type Culture Collection, Manassas, Va.; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CNRMA, National Reference Center for Mycoses and Antifungals, Pasteur Institute, Paris, France.
[c] NA, information not available.

L. tompkinsii, 6 *Pyrenochaeta* spp., 7 *Exophiala jeanselmei*, and 4 *Curvularia lunata* strains. Type strains were included for five of seven species. Strains from the CNRMA were kept at −80°C as either spore suspensions in 40% glycerol for sporulating species or mycelium in skimmed milk for nonsporulating species. During the study period, all strains were maintained by serial transfer onto agar slants every 2 to 3 months. Before testing, strains were subcultured twice onto Sabouraud's glucose agar at 27°C to ensure purity and viability.

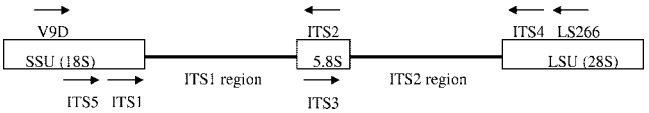

FIG. 1. Schematic representation of the rRNA gene region and positions of primers used in the study. SSU, small subunit; LSU, large subunit.

Sequencing and comparison. (i) DNA extraction. Strains were cultured in 20 ml of RPMI 1640 medium with L-glutamine but without sodium bicarbonate (Sigma-Aldrich, Saint Quentin Fallavier, France) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich). After 3 to 14 days of growth at 30°C under agitation (100 rpm), the mycelium was transferred into a tube and washed in 40 ml of sterile distilled water. Mycelium was then stored at −20°C until use. DNA extraction was done by a glass bead lysis method. Briefly, 100 mg of mycelium was homogenized for 1 min in a tube containing 1 ml of lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 10 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA [pH 8]), three 0.5-cm-diameter glass beads (Sigma), and approximately 500 mg of 425- to 600-μm glass beads (Sigma). The homogenized mycelia were then snap-frozen in liquid nitrogen, thawed, and refrozen once. DNA extraction was then done with the DNeasy plant kit (QIAGEN) according to the manufacturer's instructions.

(ii) Amplification. The genomic rRNA gene was amplified by PCR with primers V9D (5′-TTAAGTCCCTGCCCTTTGTA-3′) and LS266 (5′-GTAGTCAT ATGCTTGTCTC-3′) (11). In the case of negative amplification with V9D and LS266, other fungal universal primers (20) were used (Fig. 1). Primers were synthesized by Proligo Primers and Probes (Paris, France). Reaction volumes of 20 μl contained 1 μl of genomic DNA, 1.25 U of AmpliTaq gold (Roche), 2 μl of 10× PCR buffer (Roche), 2 μl of 25 mM $MgCl_2$ (Roche), 2 μl of 2.5 mM deoxynucleoside triphosphate, and 1 μl of each 10 μM concentrated primers. The PCR products were amplified in an ICycler thermocycler (Bio-Rad) set up with a first cycle of denaturation for 10 min at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s, with a final extension step of 10 min at 72°C. PCR products were visualized on an agarose gel after ethidium bromide staining. After purification of PCR products on P100 Gel Fine (Bio-Rad), both strands were sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (Applied Biosystems, Foster City, CA), with the primers V9D and LS266. Reaction products were analyzed using an ABI Prism 3700 automated DNA analyzer (Applied Biosystems).

(iii) PCR-restriction fragment length polymorphism (RFLP). PCR amplifications were carried out with primer pair ITS4/ITS5 (Fig. 1) by using the same protocol described above. After purification (QIAquick purification kit; QIAGEN), PCR products were digested with endonuclease SmaI for 1 h at 27°C. Restriction fragments were visualized on a 3% agarose gel after ethidium bromide staining.

Analysis of the results. Sequences were edited and manually corrected with Chromas, version 2.24 (Technelysium, Helensvale, Queensland, Australia). Multiple-sequence alignment was carried out using ClustalW 1.8. Phylogenetic trees were constructed by the neighbor-joining method using the Phylip package (http://www.infobiogen.fr) and visualized using Treeview. *Pseudallescheria boydii* was selected as the outgroup (GenBank accession number AY228119). In some instances, sequences were used for a BLAST search in GenBank.

Nucleotide sequence accession numbers. Sequences for all strains identified to the species level in the present study have been deposited in GenBank under the accession numbers listed in Table 1.

RESULTS

Sequence of the ITS1-5.8S-ITS2 region. The amplified DNA fragments of the ITS region (with V9D and LS266 primers) were obtained for 50 strains. For four strains of *Madurella mycetomatis* (IP 582.60, IP 584.78, IP 592.74, and IP 1137.76), the amplification of the whole ITS1-5.8S-ITS2 region failed despite the use of several primer sets (i.e., V9D/LS266, ITS1/ITS4, and ITS5/ITS4), but amplification of the ITS2 region was obtained with primers ITS3 and ITS4 (Fig. 1).

For comparisons, all the sequences were shortened (starting with the sequence of the ITS5 primer and ending with the sequence of the ITS4 primer). Interestingly, for most species, the lengths of the sequences were similar between strains of a given species (variations of 2 to 34 bp), except for the six strains of *Pyrenochaeta* spp. (the length ranged from 977 to 1,327 bp) and for the six strains of *Madurella grisea* (the length ranged from 951 to 1,327 bp).

Intraspecific variability of the ITS region. Results for intraspecific variability of the whole ITS1-5.8S-ITS2 region are

TABLE 2. Intraspecific variation of the ITS region according to fungal species

Species	Strain[a]	No. of bp differences			% Similarity[c]
		ITS1	5.8S	ITS2	
Madurella mycetomatis	CBS 109801^T				
	IP 582.60	NA[b]	NA	12	NA
	IP 584.78	NA	NA	12	NA
	IP 591.78	0	0	0	100
	IP 592.74	NA	NA	12	NA
	IP 595.60	33	1	18	89.8
	IP 599.60	33	1	18	89.8
	IP 1137.76	NA	NA	12	NA
	IP 1175.79	0	0	0	100
	IP 1435.83	0	0	0	100
	IP 2299.95	0	0	0	100
	IP 2303.95	64	3	51	76.9
	CNRMA 03.683	0	0	0	100
	CNRMA 03.978	0	0	0	100
	CNRMA 03.1151	0	0	0	100
Leptosphaeria senegalensis	IP 614.60^T				
	IP 610.60	1	0	1	99.6
	IP 611.60	1	0	0	99.8
	IP 612.60	1	0	0	99.8
	IP 615.60	1	0	1	99.6
	IP 617.60	1	0	0	99.8
	IP 618.60	1	0	1	99.6
	IP 619.60	1	0	0	99.8
	IP 1092.74	0	0	0	100
	IP 1155.77	2	0	1	99.4
	IP 1259.81	1	0	1	99.6
	IP 1766.88	1	0	1	99.6
Leptosphaeria tompkinsii	IP 559.60^T				
	IP 1151.76	0	0	1	99.8
	IP 1156.77	0	0	1	99.8
	CNRMA 04.1034	0	0	1	99.8
Exophiala jeanselmei	IP 71.52^T				
	IP 69.52	28	0	21	90.9
	IP 70.52	0	0	0	100
	IP 1535.84	28	0	21	90.9
	IP 1792.88	6	0	0	98.9
	IP 1908.90	28	0	21	90.9
	IP 2118.92	28	0	21	90.9
Curvularia lunata	IP 613.60				
	IP 1417.82	2	0	5	98.6
	IP 1477.83	41	0	11	89.3
	IP 2328.95	3	0	4	98.6
Pyrenochaeta romeroi	IP 862.63^T				
	IP 571.61	0	0	0	100
	IP 888.65	2	0	1	99.4

[a] IP, Pasteur Institute Collection of Fungi, Pasteur Institute, Paris, France; ATCC, American Type Culture Collection, Manassas, Va.; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CNRMA, National Reference Center for Mycoses and Antifungals, Pasteur Institute, Paris, France. T, type strain. Other designations of strains are presented in Table 1.

[b] NA, information not available.

[c] Percent similarity for the whole ITS1-5.8S-ITS2 region.

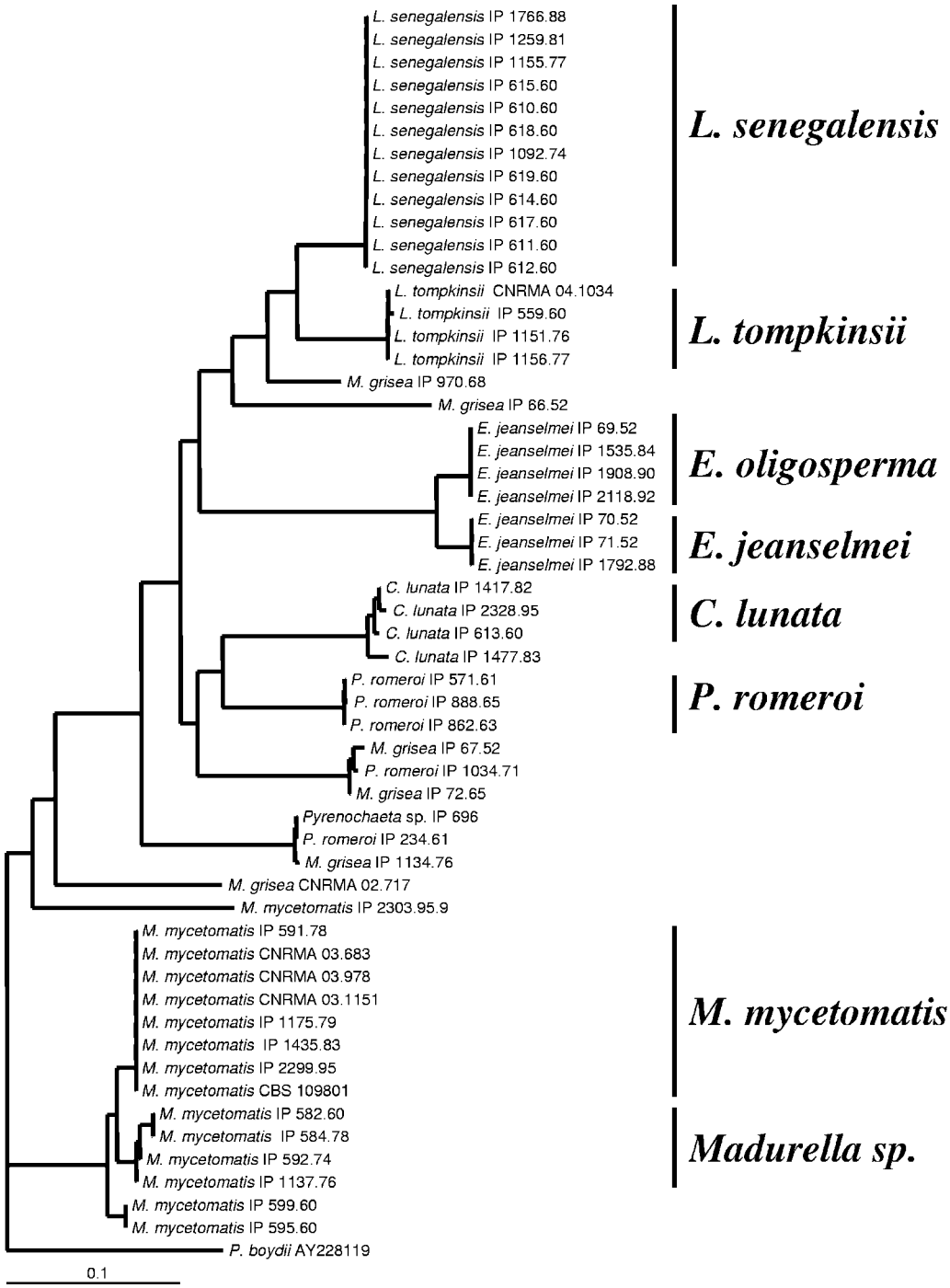

FIG. 2. Distance tree of ITS2 rRNA gene sequences of 54 strains belonging to species known as black-grain mycetoma agents. The tree was constructed using the neighbor-joining method with the Phylip software package and visualized with TreeView. *Pseudallescheria boydii* (GenBank accession number AY228119) was selected as the outgroup. CNRMA, National Reference Center for Mycoses and Antifungals, Pasteur Institute, Paris, France; IP, Pasteur Institute Collection of Fungi, Pasteur Institute, Paris, France; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

presented in Table 2. Among the 15 strains of *M. mycetomatis*, the sequences obtained for 7 strains were identical to that of the type strain. Nevertheless, the sequences for three strains (IP595.60, IP599.60, and IP2303.95) exhibited differences (77 to 90% sequence similarities) with that of the type strain. For these three strains, no close match was found in GenBank. Four strains could be analyzed only in the ITS2 region due to probable polymorphisms within the small-subunit rRNA gene. These four strains showed differences of 12 bp within the ITS2 region compared to the type strain, and the closer match in

TABLE 3. Summary of intra- and interspecies sequence similarities of the ITS1-5.8S-ITS2 regions

Species	Range of sequence similarity (%)[a]		
	Within species	Between species	Between genera
Madurella mycetomatis	100	62–71	62–71
Leptosphaeria senegalensis	99–100	64–87	64–74
Leptosphaeria tompkinsii	99–100	62–87	62–74
Pyrenochaeta romeroi	99–100	61–80	61–80
Exophiala jeanselmei	99–100	62–91	62–71
Exophiala oligosperma	100	61–91	61–71
Curvularia lunata	99–100	60–79	60–79

[a] Strains not identified to the species level were not included.

GenBank database was *M. mycetomatis*, with 96% similarity over 347 bp. These four strains were tentatively identified as *Madurella* sp. Within the 12 strains of *L. senegalensis*, differences of 2 to 3 bp were found with overall similarities of >99%. Similar results were obtained for the four strains of *L. tompkinsii*, with a sequence similarity of >99% between strains. Among the *E. jeanselmei* strains, two strains showed a sequence similarity of >98% with the type strain, while four other strains (all sharing identical sequences) showed a sequence similarity of <91% with the type strain. These four strains shared 99% sequence similarity with the *Exophiala oligosperma* type strain (8).

Within the four *C. lunata* strains, similarities of >98% were seen, except for one strain (IP 1477.83) that showed differences of more than 50 bp.

Interspecies variability of the ITS region. Figure 2 depicts a phylogenetic tree of the ITS2 region for all the strains included in the study. Alignment of the whole ITS1-5.8S-ITS2 region, excluding the four strains of *M. mycetomatis* for which sequences of the ITS1 region were not available, showed similar clustering of strains (data not shown). Except for *M. grisea*, each species was clearly resolved (Fig. 2), with sequence similarities of <88% between *L. senegalensis* and *L. tompkinsii* and <92% between *E. jeanselmei* and *E. oligosperma*. All other species shared less than 80% sequence similarity. Overall results for intra- and interspecific variability, based on the similarity matrix obtained by a comparison of sequences for all strains identified to the species level, are summarized in Table 3. The RFLP pattern obtained after digestion of PCR products with the restriction enzyme SmaI is shown in Fig. 3 for the two species of *Leptosphaeria*. Two bands were observed, indicating one restriction site for *L. senegalensis*, whereas one band was seen (i.e., no restriction site) for strains of *L. tompkinsii*.

Percentage of similarities of the ITS1-5.8S-ITS2 sequences for strains of *Pyrenochaeta* spp. and *Madurella grisea*. Among the 12 strains formerly identified as either *P. romeroi* or *M. grisea*, marked sequence heterogeneity was evidenced. The percentages of similarities of the ITS1-5.8S-ITS2 sequences and the phylogenetic relationships between these strains are presented in Table 4 and Fig. 2, respectively. Two strains of *P. romeroi* had sequences that were highly similar (>99%) to that of the type strain. In contrast, among the nine other strains, two clusters were visible, both including strains identified as *Pyrenochaeta* spp. and *M. grisea* (Table 4 and Fig. 2). The first cluster included *M. grisea* IP 1134.76, *P. romeroi* IP 234.61, and *Pyrenochaeta* sp. strain IP 696, and the second cluster included *M. grisea* IP 67.52, *M. grisea* IP 72.65, and *P. romeroi* IP 1034.71. As shown in Table 4, within each cluster, strains shared >98% sequence similarity, but the two clusters were distantly related (Fig. 2). Three other strains of *M. grisea* (CRNMA 02.717, IP 970.68, and IP 66.52) shared less than 76% sequence similarity (Table 4) and did not cluster with any other strains (Fig. 2). None of these nine strains showed significant sequence alignment with known species in GenBank except for CNRMA 02.717, which shared 96% similarity with *Phomopsis* species.

DISCUSSION

As mentioned previously (4), the genus/species-level identification of the fungi responsible for black-grain mycetomas is hampered by their frequent failure to produce characteristic diagnostic structures in culture. A reliable tool is thus needed to identify strains responsible for eumycetoma to the species level. The present study confirms the usefulness of ITS sequencing for species identification and determination of the taxonomic position of these poorly culturable fungi. Collection strains of *Madurella mycetomatis*, *Leptosphaeria* spp., *Exophiala jeanselmei*, *Curvularia lunata*, *Pyrenochaeta* spp., and *M. grisea* from various geographical origins along with the type strains, when available, were sequenced. Strains belonging to some of the species had a high degree of sequence similarity, suggesting that the species description is robust. In contrast, others (*Pyrenochaeta* spp. and *M. grisea*) were highly variable, indicating either that these species are heterogeneous or that some of the collection strains have been misidentified.

The homogeneity of *M. mycetomatis* has been reported previously, at least in Sudan (3). By sequencing the ITS region with the ITS4 and ITS5 primers (3) and using large-scale random amplification of polymorphic DNA (1), those authors suggested that this fungus had a clonal origin. Our results confirmed that some strains of *M. mycetomatis* are very similar, with identity over ca. 600 bp in the ITS1 and ITS2 regions. However, a comparison of the ITS2 regions showed that other

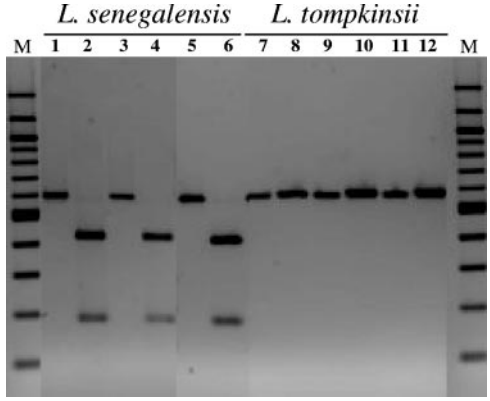

FIG. 3. RFLP analysis of three strains of *Leptosphaeria senegalensis* (lanes 1 and 2, IP 1155.77; lanes 3 and 4, IP 614.60; lanes 5 and 6, IP 612.60) and three strains of *L. tompkinsii* (lanes 7 and 8, IP 1151.76; lanes 9 and 10, IP 559.60; lanes 11 and 12, IP 1156.77). After PCR amplification using primers ITS4 and ITS5 (lanes 1, 3, 5, 7, 9, and 11), restriction profiles were generated with the endonuclease SmaI (lanes 2, 4, 6, 8, 10, and 12). M, 100-bp DNA ladder.

TABLE 4. Matrix of similarities of strains initially identified as *Pyrenochaeta romeroi* or *Madurella grisea*[a]

Strain	% Similarity to strain:								
	P. romeroi IP 1034.71	*M. grisea* IP 67.52	*M. grisea* IP 72.65	*M. grisea* IP 1134.76	*Pyrenochaeta* sp. strain IP 696	*P. romeroi* IP 234.61	*M. grisea* IP 970.68	*M. grisea* CNRMA 02.717	*M. grisea* IP 66.52
P. romeroi IP 862.63[T]	47.2	47	47.2	62.3	62.3	62.3	77	67.7	70.7
P. romeroi IP 1034.71	100	98.4	99.8	56.2	56.2	56.2	43.9	43.2	42.3
M. grisea IP 67.52		100	98.4	56.5	56.5	56.5	43.6	43.3	42.5
M. grisea IP 72.65			100	56.4	56.4	56.4	43.9	43.4	42.5
M. grisea IP 1134.76				100	99.9	99.9	59.4	58.8	58.3
Pyrenochaeta sp. strain IP 696					100	100	59.4	58.8	58.3
P. romeroi IP 234.61						100	59.4	58.8	58.3
M. grisea IP 970.68							100	66.9	76.4
M. grisea CNRMA 02.717								100	66.4
M. grisea IP 66.52									100

[a] Two strains of *P. romeroi* with sequences that were highly similar (>99%) to that of the type strain were not included. T, type strain.

strains diverged significantly from the type strain. Nevertheless, using the 15 strains studied, we were unable to find any correlation between these latter strains and a specific phenotypic trait in culture or a geographical origin. Moreover, our inability to amplify the ITS1 regions of four strains suggests the presence of mismatches in the sequences targeted by several universal fungal primers. Either these strains belong to another species of *Madurella* or *M. mycetomatis* is not as homogenous as previously reported. Indeed, the same group that suggested a clonal origin of *M. mycetomatis* strains from Sudan (1) has recently reported polymorphism within this species by using amplified fragment length polymorphism. Those authors even suggested a possible relationship with clinical data such as lesion size (19). In the present study, identical ITS sequences were found in strains recovered from different geographical origins (Sudan, Morocco, and Niger). One explanation is that some genotypes are conserved across remote geographic areas. Further studies, including extensive evaluation of environmental sources, are needed to answer this question. Because of the late onset of clinical manifestations, another explanation is that some patients were migrant workers diagnosed with mycetoma in a country distant from their country of origin. This is obviously the case for patients consulting in European countries, but it can also be true in the African continent, as migration for job searching is common. This underlines the need for well-designed epidemiological studies with detailed information and not only passive notification of the cases.

For the two *Leptosphaeria* species, i.e., *L. senegalensis* and *L. tompkinsii*, the intraspecies sequence similarity was >99% over ca. 600 bp, but the two species shared <88% sequence similarity. They were also easily distinguished by the digestion pattern of the PCR product using the restriction enzyme SmaI. Only *L. senegalensis* has an SmaI site in the ITS1 region, compared with *L. tompkinsii*. Our study does not allow conclusions on the clonal origin of these species, on the spread of a given genotype through Senegal, or on the higher virulence of a given genotype. As for *M. mycetomatis*, other studies that include clinical and environmental strains from geographical areas other than Senegal are needed before definitive conclusions can be drawn.

For the seven *E. jeanselmei* strains, two clusters were individualized with approximately 10% of differences over ca. 650 bp. Four of these strains (including one isolated from a mycetoma lesion diagnosed in West Africa) were then reidentified with confidence as *E. oligosperma*, a recently described species of *Exophiala* (8). Among the four *C. lunata* strains, one showed >10% differences within its sequence compared to the three other strains and was tentatively identified as a *Curvularia* species.

The results for *Pyrenochaeta* spp. and *M. grisea* were somewhat different from those found for the other species studied. Indeed, major differences in the ITS sequences were observed for strains belonging to these species. Comparison of the ITS sequences showed that some strains initially identified as *M. grisea* were closer to some *P. romeroi* strains than to other *M. grisea* strains. Only three strains were thus identified with confidence as *P. romeroi*, as they clustered together and with the type strain of this species. The first explanation for these discrepancies is that some of the collection strains have been misidentified. Indeed, the microscopic appearance of *Pyrenochaeta* spp. and *M. grisea* is mostly a sterile mycelium, and therefore, fruiting structures that could allowed a precise identification (7) are missing. The second explanation is that *Pyrenochaeta* spp. and *M. grisea* are very heterogeneous species. The sequences obtained for *Pyrenochaeta* spp. and *M. grisea* did not match with any other species of our study (*M. mycetomatis*, *Leptosphaeria* spp., *Exophiala* spp., and *C. lunata*), and it was impossible to cluster them in a phylogenetic tree (Fig. 2). For most of these strains, no sequence with a close match was found in GenBank, and therefore, their identification remains to be determined. Finding the specific cultural requirements necessary to obtain fructifications of these species seems unrealistic.

Overall, the results of the present study demonstrate that ITS sequencing is a useful molecular tool for reliable and rapid identification of most black-grain mycetoma agents and can be used for DNA bar coding of this group of fungi. DNA bar coding (i.e., species identification by sequencing short DNA regions) (12) using ITS regions has previously been used for plants (14) and has been successfully employed for different groups of fungi (8, 13, 16).

The taxonomic positions of some species such as *M. grisea* need improvement, and the identification of new species responsible for eumycetoma is warranted. Indeed, our findings support previous suggestions of diversity among agents of mycetoma (6). It should be kept in mind that, indeed, mycetomas

are probably a rather unspecific response to the subcutaneous inoculation of a wide range of principally saprophytic agents (5). The molecular identification of the fungi responsible for eumycetoma will allow a reassessment of the epidemiology of black-grain mycetoma. Moreover, these molecular approaches are promising for setting up new tools for the diagnosis of mycetoma agents directly from infected tissues, as previously described (2), and should help clarify the therapeutic efficacy of new antifungals according to the species responsible for black-grain mycetoma.

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